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Autoantibody Induction by DNA-Containing Immune Complexes Requires HMGB1 with the TLR2/MicroRNA-155 Pathway

Zhenke Wen,* Lin Xu,* Xi Chen,* Wei Xu,† Zhinan Yin,‡ Xiaoming Gao,† and Sidong Xiong*∗†

Anti-dsDNA Ab is reported to be the central pathogenic autoantibody involved in systemic lupus erythematosus (SLE) pathogenesis. However, the mechanisms involved in anti-dsDNA Ab production remain unclear. Recent evidence indicated that DNA-containing immune complexes (ICs) in circulation (termed “circulating DNA-containing ICs”), which are one of the hallmarks of SLE, might be involved in autoantibody production. In this study, we explored their potential role in anti-dsDNA Ab production and the underlying mechanisms in patients with SLE. We demonstrated that circulating DNA-containing ICs were able to induce anti-dsDNA Ab. Of note, HMGB1 in circulating DNA-containing ICs was crucial for anti-dsDNA Ab induction. The HMGB1 content of circulating DNA-containing ICs also correlated positively with anti-dsDNA Ab production in patients with SLE. Further, we revealed that the TLR2/MyD88/microRNA-155 (miR-155) pathway was pivotal for HMGB1 to confer anti-dsDNA Ab induction, and Ets-1 was a functional target of miR-155 in the induction of anti-dsDNA Ab by circulating DNA-containing ICs. Finally, we validated the expression of miR-155 and Ets-1 and their correlation with anti-dsDNA Ab production in patients with SLE. To our knowledge, this is the first report of the crucial role of HMGB1 in autoantibody production mediated by the TLR2/MyD88/miR-155/Ets-1 pathway. These findings identify a novel mechanism to account for the persistent production of anti-dsDNA Ab in SLE and a clue for developing a novel therapeutic strategy against SLE. The Journal of Immunology, 2013, 190: 5411–5422.

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ystemic lupus erythematosus (SLE), which is the prototypic systemic autoimmune disease, can affect virtually any organ system (1). The overall prevalence of SLE in the general population is 1 in 2000, with a predilection for women; this results in tremendous health care and societal costs because affected individuals are typically young and can suffer significant morbidity and early mortality (1). The pivotal immunologic disturbance in SLE is the formation of autoantibody that represents a major diagnostic feature of SLE and can provide clues to pathological processes in various tissues (1, 2). Of note, autoantibody directed against dsDNA, which can be present prior to clinical symptoms of SLE, is implicated in the pathogenesis of lupus nephritis and is a major cause of morbidity and mortality in SLE (1–4). In fact, anti-dsDNA Ab was reported as the central pathogenic autoantibody involved in SLE pathogenesis (5–9). However, the underlying mechanisms involved in the induction of anti-dsDNA Ab in patients with SLE remain unknown.

It has been known for some time that DNA-containing immune complexes (ICs) in the circulation (termed “circulating DNA-containing ICs”) are one of the hallmarks of SLE (10). DNA-Ab binding and subsequent events, such as complement activation, IC deposition, and cytokine release, take place in the circulation of SLE patients (10). Accumulating studies showed that circulating DNA-containing ICs could activate human plasmacytoid dendritic cells to induce type I IFN responses and, thus, are involved in SLE pathogenesis (11–13). In contrast, the potential role of circulating DNA-containing ICs in the induction of anti-dsDNA Ab in patients with SLE remains largely unexplored. Of interest, recent studies showed that DNA-containing ICs could activate transgenic rheumatoid factor B cells through a process that involves BCR and TLR9 (14–16). These findings indicated a potential role for circulating DNA-containing ICs in autoantibody production in SLE patients. Consistently, studies of SLE patients revealed that exposure of healthy monocytes to SLE serum resulted in the generation of dendritic cells that were endowed with a unique ability to promote IgG and IgA plasmablast differentiation (17–19). Combining these findings prompted us to hypothesize that the SLE plasma, especially circulating DNA-containing ICs, might induce anti-dsDNA autoantibody and, thus, maintained production of anti-dsDNA autoantibody in SLE patients.

To test our hypothesis, we explored the potential role of circulating DNA-containing ICs in anti-dsDNA Ab production and the underlying mechanisms in patients with SLE. We showed that circulating DNA-containing ICs were able to induce anti-dsDNA Ab. Of importance, we revealed that HMGB1 in circulating DNA-containing ICs was crucial for anti-dsDNA Ab induction, at least in part.
through the TLR2-mediated and MyD88-dependent miR-155/Ets-1 pathway. Our findings provided a novel mechanistic explanation for how anti-dsDNA Ab is maintained in patients with SLE and suggested that HMGB1 is a promising target for developing a therapeutic strategy to control SLE.

Materials and Methods

Patients and healthy controls

This study was approved by the Ethics Committee of Fudan University. A total of 165 SLE patients was recruited, and 6 ml peripheral blood was obtained from each person. All of the peripheral blood samples were collected from SLE patients after obtaining informed consent. The diagnosis of SLE was established according to the 1982 revised American College of Rheumatology criteria. Disease activity was evaluated using the SLE Disease Activity Index score (SLEDAI). Lupus nephritis was diagnosed with renal biopsy. Patients who had other autoimmune diseases were excluded. All patients were female and positive for plasma anti-dsDNA Ab. The mean age of the patients was 29 ± 13 y, and 16 healthy individuals matched for gender and age (±2 y) were recruited as controls.

Cell isolation

PBMCs were isolated from heparinized peripheral venous blood using a Ficoll-Hypaque gradient (GE Healthcare, Piscataway, NJ). PBMCs were washed with PBS and resuspended in RPMI 1640 media supplemented with 10% FCS and 1% glutamine/penicillin/streptomycin.

Isolation of DNA and ICs and treatment of plasma with nucleases and proteases

DNA was isolated from SLE plasma using a DNA Isolation Kit (Roche), as described previously (10). The final A260/A280 for all DNA preparations was >1.8. Less than 0.01 U/μg endonuclease was present in any of the DNA samples, based on a Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD). Isolation of ICs and treatment of plasma with nucleases and proteases were performed as previously described (20). Circulating ICs were isolated from patient plasma using the IgG Spin Purification Kit (Pierce), according to the manufacturer’s instructions. Briefly, SLE plasma was added into the spin columns containing the purification support, and IgG was eluted by centrifuge. The presence of DNA was confirmed on a 1% agarose gel, as previously described (20). To test the role of DNA in the stimulatory activity of test plasma, 20 μl patient plasma or ICs were treated with DNase I (Ambion) for 30 min at room temperature before stimulation of cells. Protease treatment of test plasma or ICs was achieved by incubation with immobilized papain (Pierce) at room temperature prior to stimulation of cells.

Cell incubation and reagents

The PBMCs (2 × 10⁶/ml) were incubated with the conditioned medium containing 20% SLE plasma (v/v) as previously described (21). Specifically, PBMCs from SLE patients were stimulated with the conditioned medium containing 20 μl self-plasma or the isolated ICs for 5 d and then assayed for supernatant anti-dsDNA Ab. A box peptide was synthesized by ChinaPeptides, Recombinant human HMGB1 protein was purchased from HMGBiotech. Recombinant human RAGE-Fc chimera was purchased from R&D Systems. TLR2/TLR4 inhibitor (OxPAPC), polyclonal Ab to human TLR2, polyclonal Ab to human TLR4, TIR (Toll/IL-1R) domain-containing adapter inducing IFN-β (TRIF) inhibitory peptide, MyD88 inhibitory peptide, and the control peptide were all purchased from InvivoGen. All reagents were used according to the manufacturer’s instructions.

RNA interference and plasmid

The validated small interfering RNA (siRNA) against human RAGE, TLR2, TLR4, or Ets-1 or control siRNA was used as previously described (22, 23). Human Ets-1 expression vector was constructed as previously described (24). Transfection of PBMCs was performed using an Amaza Cell Line Nucleofector Kit, according to the manufacturer’s instructions.

Real-time RT-PCR

Quantitative real-time RT-PCR was performed as previously described (25). The primers and probes were obtained from Applied Biosystems. Total RNA was extracted using TRIzol reagent. cDNA was synthesized with the Pri-meScript RT Reagent Kit (TaKaRa). Quantitative RT-PCR analyses were carried out to detect mRNA expression using SYBR Premix Ex Taq (TaKaRa), and GAPDH was used as an internal control. TaqMan micro-RNA assays (Applied Biosystems) were used to quantify the expression levels of mature miR-155, and U6 small nuclear RNA was used as an internal control.

Western blot analysis

Cells were lysed with M-Per Protein Extraction Reagent (Pierce) supplemented with a protease inhibitor mixture. Cytoplasmic and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce). After centrifugation at 13,000 × g at 4°C for 15 min, the supernatants were collected, and the protein concentration of the extracts was measured by BCA Protein Assay (Pierce), according to the manufacturer’s instructions. An equal amount of the total protein was loaded onto 10% SDS–polyacrylamide gels and transferred for 90 min at 100 V onto polyvinylidene fluoride membranes using a wet transfer system. The membranes were washed in 5% skim milk in PBS plus 0.05% Tween 20 for 1 h to block nonspecific protein binding sites on the membrane. Immunoblotting was performed using mAbs to HMGB1, Ets-1, and GAPDH (Cell Signaling Technology) at a dilution of 1:1,000 in nonfat milk Tris buffer. The membrane was then washed in PBS plus 0.05% Tween 20, probed with a secondary anti-rabbit Ab conjugated to HRP (Amersham Life Sciences) at a dilution of 1:5,000, developed using an ECL Western Blotting Kit (Pierce), and exposed to x-ray film (Kodak). For some experiments, one representative set of data or the cumulative data for the relative intensity are shown. To analyze the HMGB1 content of circulating DNA-containing ICs, the intensity of HMGB1 in one patient was normalized to “1,” and the content of HMGB1 in other patients was represented as their relative ratio.

Human anti-dsDNA Ab measurement

Human anti-dsDNA Ab was assayed by the Human IgG anti-dsDNA ELISA Kit (Alpha Diagnostic), according to the manufacturer’s instructions.

Statistical analysis

Unpaired t test and Pearson correlation were used for statistical analyses. A p value <0.05 was considered statistically significant. All statistical analyses were performed using SPSS statistical software version 16 (SPSS).

Results

Circulating DNA-containing ICs induced anti-dsDNA Ab

To detect whether SLE plasma was endowed with stimulatory activity to induce anti-dsDNA Ab, PBMCs from SLE patients were exposed to conditioned medium containing 20% SLE plasma and assessed for the supernatant anti-dsDNA Ab. We found that the SLE plasma was effective in inducing anti-dsDNA Ab (Fig. 1A, p < 0.05). In contrast, exposure to 20% normal plasma or to control plasma (20% fetal calf plasma) resulted in no significant production of anti-dsDNA Ab (Fig. 1A, p > 0.05). Further, we showed that SLE plasma could induce anti-dsDNA Ab in a dose-dependent manner, and the dose of SLE plasma in the conditioned medium correlated positively with the supernatant level of anti-dsDNA Ab (Fig. 1B, r = +0.859, p < 0.001). Although the anti-dsDNA Ab pre-existing in SLE plasma could contribute to their supernatant level, this contribution of added anti-dsDNA Ab had no significant effect on the dose-dependent manner of SLE plasma in the induction of anti-dsDNA Ab (Supplemental Fig. 1). Based on these results, exposure to conditioned medium containing 20% SLE plasma was used for the subsequent experiments. These findings demonstrated that SLE plasma was endowed with stimulatory activity and induced anti-dsDNA Ab.

To characterize whether circulating DNA-containing ICs were required for the stimulatory activity, SLE plasma was pretreated with DNase and then incubated with the PBMCs of SLE patients. As shown in Fig. 1C, we found that pretreatment of SLE plasma with DNase abrogated the induction of anti-dsDNA Ab in a dose-dependent manner (p < 0.05), suggesting that the DNA component was required for the stimulatory activity of SLE plasma. However, when PBMCs of SLE patients were incubated with DNA alone isolated from SLE plasma, we found that DNA isolated from 20% SLE plasma could not induce anti-dsDNA Ab.

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FIGURE 1. Circulating DNA-containing ICs were able to induce anti-dsDNA Ab. (A) PBMCs of SLE patients were incubated with conditioned medium containing 20% of the indicated plasma for 5 d. (B) PBMCs of SLE patients were incubated with conditioned medium containing the indicated dose of SLE plasma for 5 d. (C) SLE plasma was pretreated with the indicated dose of DNase. PBMCs of SLE patients were incubated with conditioned medium containing 20% of the pretreated SLE plasma for 5 d. (D) PBMCs of SLE patients were incubated with conditioned medium containing the DNA isolated from 20, 40, or 60% of SLE plasma for 5 d. (E) SLE plasma was pretreated with proteinase K (100 μg/ml) at 37°C for 3 h. PBMCs of SLE patients were incubated with conditioned medium containing 20% of the pretreated SLE plasma for 5 d. (F) SLE plasma was pretreated with the indicated dose of papain. PBMCs of SLE patients were incubated with conditioned medium containing 20% of the pretreated SLE plasma for 5 d. (G) Isolated ICs were pretreated or not with DNase (1 U/μl) plus papain (20 μl) and then incubated with PBMCs of SLE patients for 5 d. (H) PBMCs of SLE patients were incubated with isolated DNA plus Ig for 5 d. (I) DNA was isolated from 20% of SLE plasma, and Ig was isolated from 20% of SLE plasma pretreated with DNase. Data represent the average from experiments performed in triplicates for each patient. Each symbol represents the data from one SLE patient. *p < 0.05.

HMGB1 in circulating DNA-containing ICs was crucial for anti-dsDNA Ab induction

Previous studies showed that HMGB1 was a part of circulating ICs in the peripheral blood of SLE patients (21, 26). Recent studies showed that HMGB1 was an essential component of DNA-containing ICs that stimulated cytokine production (21, 27). Thus, we explored the potential role of HMGB1 in the production of anti-dsDNA Ab induced by circulating DNA-containing ICs. We confirmed that HMGB1 was readily detected in the isolated ICs from SLE patients (Fig. 2A). Then, the PBMCs of SLE patients were incubated with isolated ICs in the presence of A box peptide, and we found that blockade of HMGB1 using A box peptide inhibited the production of anti-dsDNA Ab, which could be abrogated by pretreatment with DNase and papain (p < 0.05). These data demonstrated that circulating DNA-containing ICs were able to induce anti-dsDNA Ab. However, we noticed that when immobilized papain was used in the culture system, the level of anti-dsDNA Ab was still higher than that induced either by the isolated DNA alone or by proteinase K–pretreated SLE plasma, indicating that another protein might be involved in anti-dsDNA Ab induction. To confirm this phenomenon, PBMCs of SLE patients were incubated with the DNA isolated from SLE plasma plus Ig isolated from DNase-pretreated SLE plasma. Compared with the isolated ICs, we found a significantly lower level of anti-dsDNA Ab in response to the isolated DNA plus Ig (Fig. 1I, p < 0.05). These results suggested that another protein component in circulating DNA-containing ICs was required for anti-dsDNA Ab induction.

DNA was isolated from 20% of SLE plasma, and Ig was isolated from 20% of SLE plasma pretreated with DNase. Data represent the average from experiments performed in triplicates for each patient. Each symbol represents the data from one SLE patient. *p < 0.05.
anti-dsDNA Ab (Fig. 2D, \( p < 0.05 \)). In addition, we confirmed that A box peptide also abrogated the anti-dsDNA Ab induction by conditioned medium containing 20% SLE plasma in a dose-dependent manner (Fig. 2E, \( p < 0.05 \)). Our findings strongly demonstrated that HMGB1 in circulating DNA-containing ICs was crucial for anti-dsDNA Ab induction.

**HMGB1 content of circulating DNA-containing ICs correlated positively with anti-dsDNA Ab production in patients with SLE**

To evaluate the possible role of HMGB1 in the induction of anti-dsDNA Ab in vivo, the HMGB1 in circulating DNA-containing ICs was assessed and analyzed for its correlation with the serological level of anti-dsDNA Ab in SLE patients. As shown in Fig. 3A, we found that the relative intensity of HMGB1 correlated positively with the level of anti-dsDNA Ab in SLE patients (\( p < 0.05 \)). Further, the relative intensity of HMGB1 correlated inversely with the amount of complement 3 (Fig. 3B, \( p < 0.05 \)). We then analyzed the correlation between the HMGB1 content and the disease activity index and found that the relative intensity of HMGB1 correlated closely with the SLEDAI in SLE patients (Fig. 3C, \( p < 0.05 \)). Consistent with this, the relative intensity of HMGB1 was significantly higher in patients with lupus nephritis than in patients without lupus nephritis (Fig. 3D, \( p < 0.05 \)).

The crucial role of HMGB1 in anti-dsDNA Ab induction was mediated by TLR2/MyD88 signaling

Given the crucial role of HMGB1 in anti-dsDNA Ab induction and its close correlation with the serological level of anti-dsDNA Ab in vivo, we next sought to elucidate the underlying mechanism for how HMGB1 acted in anti-dsDNA Ab induction. Previous studies demonstrated that RAGE, TLR2, and TLR4 were receptors for recognition of HMGB1 (21, 27, 28). To characterize which receptor was required for anti-dsDNA Ab induction, PBMCs of SLE patients were cultured with isolated ICs in the presence of inhibitors to RAGE and TLR2/TLR4. As shown in Fig. 4A and 4B, we found that inhibition of TLR2/TLR4, but not RAGE, abrogated the induction of anti-dsDNA Ab (\( p < 0.05 \)). Further, we showed that neutralization Ab to TLR2, but not TLR4, substantially inhibited anti-dsDNA Ab production in a dose-dependent manner (Fig. 4C, \( p < 0.05 \)). We further confirmed these results using transfection with siRNA against RAGE, TLR2, and TLR4, and similar results were observed (Fig. 4D, \( p < 0.05 \)). These findings suggested that the crucial role for HMGB1 in anti-dsDNA Ab induction was mediated by TLR2, whereas TLR4 and RAGE were not essential. Given the vital role of MyD88 in TLR2 signaling, we next assessed the effect of the MyD88 pathway on the induction of anti-dsDNA Ab. Results showed that inhibition of MyD88 using the inhibitory peptide decreased anti-dsDNA Ab induction in a dose-dependent manner (Fig. 4E, \( p < 0.05 \)). In contrast, inhibition of TRIF using the inhibitory peptide had no significant effect on anti-dsDNA Ab induction (Fig. 4F, \( p > 0.05 \)). Finally, we demonstrated that blockade of TLR2 or MyD88 signaling effectively abrogated the anti-dsDNA Ab induction enhanced by HMGB1 (Fig. 4G, \( p < 0.05 \)). These findings demonstrated that TLR2/MyD88 signaling was required for the crucial role of HMGB1 in anti-dsDNA Ab induction.

**FIGURE 2.** HMGB1 in circulating DNA-containing ICs was crucial for anti-dsDNA Ab induction. (A) Isolated ICs were assessed for the presence of HMGB1 protein using Western blot. Data from one representative experiment are shown. (B) PBMCs of SLE patients were incubated with isolated ICs in the presence of the indicated dose of A box peptide for 5 d. (C) PBMCs of SLE patients were incubated with isolated ICs in the presence of the indicated dose of HMGB1 protein for 5 d. (D) PBMCs of SLE patients were incubated with isolated DNA plus Ig in the presence of HMGB1 protein (1 \( \mu \)g/ml) for 5 d. (E) PBMCs of SLE patients were incubated with conditioned medium containing 20% of SLE plasma in the presence of the indicated dose of A box peptide for 5 d. Data represent the average from experiments performed in triplicates for each patient. Each symbol represents the data from one SLE patient. *\( p < 0.05 \).
MyD88-dependent upregulation of miR-155 was pivotal for HMGB1 to confer anti-dsDNA Ab induction

miR-155, which is encoded within a region known as the B cell integration cluster gene, has been implicated in the pathogenesis of autoimmune disease (29). Recent evidence showed that miR-155 was an important regulator in Ab production (30–33). Of interest, TLR activation induced miR-155 expression through the MyD88-dependent or TRIF-dependent signaling pathways (34). Thus, we then determined whether miR-155 was involved in the induction of anti-dsDNA Ab. We found that exposure to isolated ICs resulted in the increased expression of miR-155 in PBMCs of SLE patients, which could be abrogated by the inhibitory peptide of MyD88 (Fig. 5A, p < 0.05). Further, we showed that administration with HMGB1 protein together with the isolated ICs could enhance the expression of miR-155, and this process could be abrogated by a box peptide and MyD88 inhibitory peptide (Fig. 5B, p < 0.05). These data demonstrated the MyD88-dependent upregulation of miR-155 by HMGB1. To further assess the potential role of miR-155 in the induction of anti-dsDNA Ab, PBMCs of SLE patients were transfected with miR-155 inhibitor and then incubated with isolated ICs, with or without HMGB1 protein. We found that miR-155 inhibitor transfection effectively decreased miR-155 expression (Fig. 5C, p < 0.05). Further, miR-155 inhibitor transfection abrogated the anti-dsDNA Ab production induced either by isolated ICs alone or by isolated ICs plus HMGB1 protein (Fig. 5D, p < 0.05). Consistent with that finding, miR-155 mimics transfection effectively increased miR-155 expression and enhanced the anti-dsDNA Ab production induced by isolated ICs alone or isolated ICs plus HMGB1 protein (Fig. 5E, 5F, p < 0.05). These data demonstrated that MyD88-dependent upregulation of miR-155 was pivotal for HMGB1 to confer anti-dsDNA Ab induction.

Ets-1 was a functional target of miR-155 in induction of anti-dsDNA Ab by circulating DNA-containing ICs

To further understand the effect of miR-155 on anti-dsDNA Ab induction, we predicted the target of miR-155 using prediction programs, including TargetScan and Miranda, and selected 10 possible targets, including PU.1, AICDA, ETS1, MEIS1, c-MAF, RHEB, MYB, IKBKE, BCORL1, and SOCS1 for real-time PCR analysis. We found that the expression of ETS1 in PBMCs of SLE patients decreased following exposure to isolated ICs (Fig. 6A, p < 0.05). We further confirmed the expression of Ets-1 protein using Western blot and found similar results (Fig. 6B, 6C, p < 0.05). To confirm this phenomenon, PBMCs of SLE patients were transfected with miR-155 inhibitor and then incubated with isolated ICs; we found that the mRNA and protein expression of Ets-1 was significantly increased by transfection with miR-155 inhibitor (Fig. 6D–F, p < 0.05). Given that Ets-1 is a direct target of miR-155 (35), we next sought to detect its potential role in the induction of anti-dsDNA Ab. As shown in Fig. 6G and 6H, we found that transfection with Ets-1 expression vector significantly increased the expression of Ets-1 in PBMCs of SLE patients (p < 0.05). Of note, transfection with Ets-1 expression vector significantly decreased the anti-dsDNA Ab production induced by isolated ICs (Fig. 6I, p < 0.05). Further, we revealed that transfection of Ets-1 expression vector abrogated the effect of miR-155 mimics on anti-dsDNA Ab induction (Fig. 6J, p < 0.05). Consistent with this, transfection of Ets-1 siRNA abrogated the effect of miR-155 inhibitor on anti-dsDNA Ab induction (Fig. 6K, p < 0.05). Of note, transfection with Ets-1 siRNA abrogated the effect of Ets-1 expression vector on anti-dsDNA Ab production (Fig. 6L, p < 0.05). These results demonstrated that Ets-1 was a bona fide target of miR-155 in the induction of anti-dsDNA Ab by circulating DNA-containing ICs.

Increased expression of miR-155 correlated positively with anti-dsDNA Ab production and inversely with Ets-1 expression in patients with SLE

To further investigate the in vivo relevance of our above findings, we assessed the expression of miR-155 and Ets-1 in PBMCs of SLE patients. As shown in Fig. 7A, we found that miR-155 expression was significantly increased in SLE patients (p < 0.05). In contrast, the expression of Ets-1 was significantly downregulated in SLE patients (Fig. 7B, p < 0.05). We noted that the expression of miR-155 correlated positively with the HMGB1 content of circulating DNA-containing ICs (Fig. 7C, p < 0.05). Further, the expression of miR-155 was associated positively with the serological level of anti-dsDNA Ab in patients with or without lupus nephritis. Data represent the average from experiments performed in triplicates for each patient. Each symbol represents one SLE patient. *p < 0.05.
anti-dsDNA Ab and inversely with the expression of Ets-1 in SLE patients (Fig. 7D, 7E, $p < 0.05$). Consistent with this, the expression of Ets-1 correlated inversely with the serological level of anti-dsDNA Ab in SLE patients (Fig. 7F, $p < 0.05$). These findings were in line with our above data that demonstrated that Ets-1 was a functional target of miR-155, which was vital for HMGB1 to confer anti-dsDNA Ab induction through TLR2/MyD88 signaling (Fig. 8).

**Discussion**

SLE is an autoimmune disease that predominantly affects females; loss of tolerance to nucleic acids and their interacting proteins results in the production of pathogenic autoantibodies that cause inflammation and tissue damage (36). Although intensive investigations on the driving forces behind the onset of SLE are necessary, equally important are the underlying mechanisms that account for its flare-ups. Three main immune pathways have been identified in SLE: aberrant clearance of nucleic acid–containing debris and ICs, excessive innate immune activation, and abnormal T and B lymphocyte activation (36, 37). Defective clearance of apoptotic particles and nucleic acid–containing ICs results in secondary necrosis and an overload of self-Ags that, instead of being safely consumed by phagocytes, access receptors, such as activating FcRs, TLRs, or BCRs, and may induce the production...
of type I IFN and the expansion of autoreactive effector cells (36). Previous studies implicated a potential role for DNA-containing ICs in the induction of autoantibody in SLE (14–16), but the precise mechanisms remain unclear. In this study, we explored the effect of circulating DNA-containing ICs on the induction of anti-dsDNA Ab, which is the central pathogenic autoantibody and is closely correlated with disease activity, in patients with SLE. We found that the circulating DNA-containing ICs were able to induce anti-dsDNA Ab. Of note, our results extend previous studies by demonstrating that HMGB1, which is a component of circulating DNA-containing ICs, was crucial for anti-dsDNA Ab induction. Although the mechanisms responsible for the formation of HMGB1-DNA-containing ICs and its structure remain to be elucidated, our results suggested that DNA-Ab binding per se might not be enough to drive the flare-ups of SLE. Instead, HMGB1 complexes with DNA-containing ICs might well be one of the driving forces behind flare-ups of SLE. Furthermore, we revealed that the TLR2/MyD88/miR-155/Ets-1 pathway was essential for HMGB1 to confer anti-dsDNA Ab induction. In addition, we found that the circulating DNA-containing ICs induced a significantly lower level of anti-dsDNA Ab from PBMCs isolated from patients without anti-dsDNA Ab than did those from patients with anti-dsDNA Ab (Supplemental Fig. 2), indicating a specificity of the anti-dsDNA response which warrants further study. These findings could fa-
ciliate our further understanding of the pathogenesis behind flare-ups of SLE and provide a clue for developing a novel strategy to control SLE.

HMGB1 is a ubiquitously expressed, abundant nonhistone chromosomal protein of 215 aa, with a highly conserved sequence across species (38). At least three receptors were reported to mediate the proinflammatory and immune-activating effects of extracellular HMGB1: RAGE, TLR2, and TLR4 (26). Stimulation of these receptors resulted in activation of the transcription factor NF-κB, inducing the transcription of multiple proinflammatory genes (26, 39). In addition, HMGB1 could act as an endogenous immune adjuvant and control T cell activation (40, 41). Previous evidence suggested that HMGB1 contributed to SLE pathogenesis as a result of its proinflammatory and immunostimulatory prop-

**FIGURE 6.** Ets-1 was a functional target of miR-155 in the induction of anti-dsDNA Ab. (A) PBMCs of SLE patients were incubated with isolated ICs for 48 h and then assayed for the mRNA expression of the indicated genes. Data represent the relative ratio of these genes induced by ICs versus the control group. (B and C) PBMCs of SLE patients were incubated with isolated ICs for 48 h and then assayed for their expression of Ets-1 protein. (D–F) PBMCs of SLE patients were transfected with miR-155 inhibitor and then incubated with isolated ICs for 48 h. The expression of mRNA and protein level of Ets-1 were determined. (G and H) PBMCs of SLE patients were transfected with Ets-1 expression vector for 48 h and then assayed for Ets-1 expression. (I) PBMCs of SLE patients were transfected with Ets-1 expression vector and then incubated with isolated ICs for 5 d. (J) PBMCs of SLE patients were transfected with miR-155 mimics plus Ets-1 expression vector or its control and then incubated with isolated ICs for 5 d. (K) PBMCs of SLE patients were transfected with miR-155 inhibitor plus Ets-1 siRNA or its control and then incubated with isolated ICs for 5 d. Data represent the average from experiments performed in triplicates for each patient. Each symbol represents the data from one SLE patient. *p < 0.05.
For example, HMGB1 was an essential component of DNA-containing ICs that stimulated cytokine production through a TLR9–MyD88 pathway involving the multivalent receptor RAGE (21). HMGB1, probably by activating its receptors TLR2 and RAGE, exhibited a synergistic effect on the upregulation of proinflammatory gene expression in renal mesangial cells in vitro and chemokine expression in kidneys of BALB/c mice (42). However, whether HMGB1 plays a role in autoantibody produc-

FIGURE 7. Expression of miR-155 and Ets-1 and their association with anti-dsDNA Ab production in patients with SLE. The expression of miR-155 (A) and Ets-1 (B) in PBMCs of SLE patients was detected using real-time PCR. (C–F) Correlation among HMGB1 content of circulating DNA-containing ICs, expression of miR-155 and Ets-1 in PBMCs, and serological anti-dsDNA Ab. Data represent the average from experiments performed in triplicates for each patient. Each symbol represents the data from one SLE patient. *p < 0.05.

FIGURE 8. Schematic diagram for anti-dsDNA Ab production. During anti-dsDNA Ab production, DNA-containing ICs could induce the activation and proliferation of autoreactive B cells via BCR and TLR9 (14–16). BCR could enhance the TLR-dependent canonical NF-κB pathway, likely through TRAF6 activation, and TLR could enhance the BCR-activated noncanonical NF-κB pathway by inducing p100 expression, thereby synergizing to cause activation-induced cytidine deaminase and class-switch DNA recombination (56). Meanwhile, HMGB1, which is a component of circulating DNA-containing ICs, could activate the TLR2/MyD88/miR-155 pathway and, thus, decrease Ets-1 expression, which contributes to plasmacytic differentiation by upregulating the activity of Blimp-1. In addition, TLR9 signaling might contribute to miR-155 expression, and TLR2 signaling might enhance the canonical NF-κB pathway during induction of anti-dsDNA Ab. Collectively, circulating DNA-containing ICs induce a positive feedback on anti-dsDNA Ab production in patients with SLE.
tion in SLE remains largely unknown. One study showed that HMGB1-containing nucleosomes from apoptotic cells induced anti-dsDNA and anti-histone IgG responses in mice in a TLR2-dependent manner (43). To our knowledge, this is the first study to report that HMGB1 was crucial for the induction of anti-dsDNA Ab in patients with SLE through the TLR2-mediated and MyD88/miR-155 pathway, whereas RAGE and TLR4 were not essential. In addition, we found that HMGB1 alone could not induce the production of anti-dsDNA Ab. Collectively, it seemed that HMGB1 was a crucial cofactor that could modify the stimulatory activity of DNA-containing ICs in the induction of proinflammation and autoantibodies and is a promising target for the development of a novel therapy against SLE.

miR-155 is encoded within a region known as the B cell integration cluster gene, which was identified originally as a frequent integration site for the avian leukemia virus (44, 45). Accumulating data suggested that miR-155 was an important regulator in Ab production (30). Bic/miR-155–deficient mice exhibited defective B immunity, including reduced numbers of germinal center B cells (31, 32), miR-155–deficient B cells resulted in reduced extrafollicular and germinal center responses and failed to produce high-affinity IgG1 Abs via targeting on PU.1 (33). We found that miR-155 was significantly upregulated by HMGB1 in a MyD88-dependent manner during the induction of anti-dsDNA Ab, which was consistent with a previous study that showed that TLR activation could induce miR-155 expression through MyD88-dependent or TRIF-dependent pathways (34). Of note, we further showed that upregulation of miR-155 was crucial for HMGB1 to confer anti-dsDNA Ab induction. We identified Ets-1 as the functional target of miR-155 during the induction of anti-dsDNA Ab. Given that the transcription factor Ets-1 could interact with Blimp-1, which leads to a block in Blimp-1 DNA binding activity and a reduction in the ability of Blimp-1 to repress target genes, and thus is a negative regulator of plasmacytic differentiation and Ab production (46), our findings suggested that miR-155 could promote plasmacytic differentiation by targeting Ets-1 in the induction of anti-dsDNA Ab.

In this study, to explore the in vivo relevance of our findings, we validated the HMGB1 content of circulating DNA-containing ICs and the expression of miR-155 and Ets-1 in PBMCs of SLE patients. We found that the HMGB1 content of circulating DNA-containing ICs correlated positively with anti-dsDNA Ab production and disease activity and inversely with the level of serum complement 3 in SLE patients. Our data were consistent with previous studies that showed that the increased concentration of serum HMGB1 correlated positively with SLE disease activity and inversely with the levels of complement components C4 and C3 (47–50). In addition, we found that miR-155 expression in PBMCs of SLE patients was significantly elevated and correlated positively with anti-dsDNA Ab production; this was in line with a previous study that showed that miR-155 was significantly increased in splenocytes from MRL-lpr mice or B6-lpr mice (51). These findings suggested that miR-155 was an important player in autoantibody production and, thus, is involved in SLE pathogenesis. Similarly, recent evidence showed that miR-155–deficient mice were highly resistant to the development of collagen-induced arthritis and experimental autoimmune encephalomyelitis (52, 53). Finally, we revealed that the expression of Ets-1 in PBMCs was significantly downregulated and correlated inversely with anti-dsDNA Ab production in SLE patients. Consistent with our results, a decreased Ets-1 mRNA level in PBMCs of SLE patients was also reported (54). Collectively, our findings in vivo were consistent with previous data, as well as our findings in vitro, which demonstrated that Ets-1 was a functional target of miR-155, which was vital for HMGB1 to confer anti-dsDNA Ab induction.

Given the accumulation of circulating DNA-containing ICs in SLE patients and their ability to induce anti-dsDNA Ab, we believe that circulating DNA-containing ICs could play an important role in anti-dsDNA Ab production in SLE. Of note, given the existence of anti-dsDNA Ab in circulating DNA-containing ICs, our findings suggested that circulating DNA-containing ICs induced a positive feedback on anti-dsDNA Ab production in SLE, which might account, at least in part, for the persistent production of anti-dsDNA Ab in SLE. Although many mechanisms have been proposed by which persistent Ab production can be maintained, they have one thing in common: continual stimulation of B cells to proliferate and differentiate into Ab-secreting plasma cells (55). Previous studies showed that DNA-containing ICs could induce the activation and proliferation of autoreactive B cells through BCR and TLR9 (14–16). A recent study showed that BCR signaling activated the noncanonical NF-κB pathway and enhanced the TLR-dependent canonical NF-κB pathway, resulting in activation-induced cytokine deaminase, which is critical for class-switch DNA recombination (56). In this study, we demonstrated that HMGB1, which is a component of circulating DNA-containing ICs, could activate the TLR2/MyD88/miR-155 pathway and, thus, decrease Ets-1 expression. We also found that administration of chloroquine could significantly inhibit the production of anti-dsDNA Ab induced by circulating DNA-containing ICs, as well as that the activity of Blimp-1, which is critical for plasmacytic differentiation and Ab secretion, was also elevated in response to stimulation with circulating DNA-containing ICs (Supplemental Fig. 3).

Therefore, as shown in Fig. 8, we presumed that, during anti-dsDNA Ab induction, DNA-containing ICs induced the activation and proliferation of autoreactive B cells via BCR and TLR9 (14–16). BCR signaling activated the noncanonical NF-κB pathway and enhanced the TLR-dependent canonical NF-κB pathway, thereby synergizing to cause activation-induced cytokine deaminase and class-switch DNA recombination (56). Meanwhile, HMGB1, which is in complex with DNA-containing ICs, activated the TLR2/MyD88/miR-155 pathway and downregulated the expression of Ets-1, which resulted in upregulated activity of Blimp-1 and promoted plasmacytic differentiation and contributed to anti-dsDNA Ab production. However, it should be pointed out that we did not exclude other factors that might be involved in the induction of anti-dsDNA Ab. It remains to be elucidated whether other components might combine with the ICs and be involved in the induction of anti-dsDNA Ab in SLE patients.

In summary, to our knowledge this is the first article to report that HMGB1 is crucial for DNA-containing ICs to induce anti-dsDNA Ab production; HMGB1 acts through the TLR2-mediated and MyD88-dependent miR-155/Ets-1 pathway. Our findings provide a novel mechanistic explanation for how the persistent production of anti-dsDNA autoantibody is maintained in SLE; this may account, at least in part, for SLE flare-ups and will be helpful for developing novel therapeutic strategies against this disease.

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Disclosures

The authors have no financial conflicts of interest.


PBMCs isolated from SLE patients were incubated with the conditioned medium containing the indicated dose of SLE plasma for 5d, and then assayed for their production of anti-dsDNA antibody. Data shown was the absolute level of induced anti-dsDNA antibody by subtracting the level of added antibody from the total level of supernatant anti-dsDNA antibody. Each dot represented the result from one SLE patient.
PBMCs isolated from the indicated SLE patients were incubated with the isolated immune complexes for 5d, and then assayed for their production of anti-dsDNA antibody. Data represented the mean (±SD) from experiments performed in triplicates for six patients in each group. *P<0.05.
Supplementary Figure 3

(A) PBMCs isolated from SLE patients were incubated with the isolated immune complexes with or without the indicated dose of chloroquine for 5d. Then the anti-dsDNA Ab in the supernatants was analyzed. Each dot represented the result from one SLE patient. (B) PBMCs isolated from SLE patients were incubated with the isolated immune complexes for 48h, and then assayed for their expression of Blimp-1 using western blot.